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Parameters evaluation of fructooligosaccharides production by sucrose biotransformation using an osmophilic *Aureobasium pullulans* strain

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Abstract

The effects of production parameters on the biotransformation of sucrose in fructooligosaccharides (FOS) by osmophilic microorganisms Aureobasidium pullulans was evaluated by the RSM (Response Surface Metodology) exactly Plackett-Burman Design with 12 variables: concentration of sucrose, inoculum, yeast extract, urea, K2HPO4, (NH4)2SO4, MgSO4, ZnSO4, MnSO4. The strategy used was PB 16 with additional 3 central points, comprising 19 experiments. The levels of each variable (%) were sucrose: 20 a 40; yeast extract: 0 a 0.5; inoculum: 1 a 20; K2HPO4: 0 – 0.0435; urea: 0 – 0.015; (NH4)2SO4: 0 – 0.033; MgSO4.7H2O: 0 – 0.0245; ZnSO4: 0.0015; MnSO4.7H2O: 0 – 0.001, pH: 4.5 a 6.0; temperature (°C): 27 a 30; agitation (rpm): 150 a 250. The high yields reach values of 54.7% at 48h of reaction in treatment 9, 49.97% at 24h for treatment 4 and 49.34% in 24h for treatment 12. The variable effects were estimated for each parameter FOS Yield and total FOS concentration. The FOS Yield data showed significance at 24h the variables agitation, with negative effect -16.32 and p-value 0.016, MnSO4, with positive effect 15.33 and p-value 0.020. At 48h the agitation was the only parameter significant with negative effect -14.94. The inoculum levels showed significance at 0h with positive effect 14.08, however it was negative at 24 and 48 hours. Regarding Total FOS Concentration, sucrose levels displayed p-values of 0.0085, 0.028 and 0.046 at 24, 48 and 72h respectively. MnSO4 was the only mineral presenting stimulatory effect upon FOS concentration added. More research should be done to evaluate and optimize this process. This in turn can provide a focus for effective use of Aureobasidium pullulans to synthesize fructooligosaccharide through bioconversion using whole cells and obtaining the product by direct manner.

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Keywords: fructooligosaccharides; biotransformation; plackett-burman design; osmophilic microrganisms; prebiotic.

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1. Introduction

The fructooligosaccharides (FOS) belonging to the prebiotics group that are "non-digestible oligosaccharides food ingredients but fermentable by the bacteria in the gut microbiota. They selectively promote growth of the beneficial bacteria (lactobacilli and bifidobacteria) and provide a series of benefits to the human health. Such effects include activation of the human immune system, maintenance the intestinal microbiota, resistance to infection, enhanced mineral absorption by the gastrointestinal tract, synthesis of B complex vitamin, lowering of serum cholesterol and preventing carcinogenic tumors [1]. Among the most important compounds found in functional foods are the oligosaccharides of the fructan type acting as bifidogenic agents and immune system stimulators associated with the intestinal mucosa [2].

Industrial production of this ingredient was mainly done using the enzyme fructosyltransferase of Aspergillus niger and Aureobasidium sp. reaching yield value around 60% and 53-59% respectively [3].

In our study, the synthesis of FOS was performed from sucrose metabolized by Aureobasidium pullulans, isolated from honeycomb. In this context this study was done through RSM (Response Surface Metodology) using a Plackett-Burman matrix with 16 assays (PB-16) to evaluate 12 variables: concentration of sucrose, inoculum, yeast extract, urea, K2HPO4, (NH4)2SO4, MgSO4, ZnSO4, MnSO4. This method is a screening approach used to statistically select the significant variables of numerous factor-experiments, focusing on a reduction in the number of trials in the final design [4].

Therefore, the objective of this study was to evaluate the impact of several variables on sucrose biotransformation into fructooligosaccharides.

2. Materials & Methods

The culture of *Aureobasidium pullulans* was maintained in YEPD slants, containing glucose 2% (w/v), yeast extract 1% (w/v), peptone 1% (w/v), agar 1.5 % (w/v) at 4°C. For the pre-inoculum cultive a loopful of cells was streaked into YEPD plate, for 48h at 30°C. This was transferred to 150 ml culture medium of cane molasses containing 6% (w/v) of total reduce sugars for 48h, 150 rpm at 30°C. The suspension was centrifuged at 10.000 rpm for 15 min and the cells were used for initial inoculum for the experiments that were done according strategy Plackett-Burman Experimental Design to identify the significant parameters in the fermentation process. According to the common parameters on the fermentation, N of the Plackett-Burman experimental design was set 16 to study 12 parameters effects, with the addition of 3 central points to estimate the experimental error. The parameters evaluated were the amount of sucrose, yeast extract, inoculum, K_2HPO_4, urea, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$, $MnSO_4.7H_2O$ and the culture conditions as pH, temperature and agitation. The factor levels and codes were shown in Table 1.

Fermentations proceed in 125 ml flasks with 20 ml of medium and the sampling were done at 0, 24, 48 and 72 hours. The growth mesuring was done by absorbance at 600 nm, and sugars by HPLC, column Lichrospher 100 NH2, 26°C, 1 ml/min with acetonitrile:water 70:30 (v:v) as mobile phase.

Table 1. Codes and levels of factors of Plackett-Burman

Independent Variables		Levels		
		-1	**0**	**+1**
X1	Sucrose (%)	20	30	40
X2	Inoculum (%)	1	10.5	20
X3	pH	4.5	5.25	6.0
X4	Temperature (°C)	28	30	32
X5	Agitation (rpm)	150	200	250
X6	Yeast extract (%)	0	0.2500	0.5000
X7	Urea (%)	0	0.0075	0.0150
X8	K_2HPO_4 (%)	0	0.0435	0.0435
X9	$(NH_4)_2SO_4$ (%)	0	0.0218	0.0330
X10	$MgSO_4.7H_2O$ (%)	0	0.0165	0.0245
X11	$ZnSO_4.7H2O$ (%)	0	0.0008	0.0015
X12	$MnSO_4.7H_2O$ (%)	0	0.0005	0.0010

3. Results & Discussion

The effects of the above 12 variables on yield, which were calculated through formula: Y= [total FOS]*100/ [initial sucrose] and concentration of total FOS % (g/100 ml) formed of the response value were studied using Plackett-Burman experimental design, and the experimental design and the results were shown in Table 2. The statistical analysis were conducted by the software STATISTICA® 7.0 considering a significant level of 10% (P<0.1).

Table 2. Program and experiments results in Plackett-Burman

Treat-ment (T)	**Variables or factors**												**0 hour**		**24 hour**		**48 hour**		**72 hour**	
	X 1	**X 2**	**X 3**	**X 4**	**X 5**	**X 6**	**X 7**	**X 8**	**X 9**	**X 10**	**X 11**	**X 12**	**FOS %**	**Yield**	**FOS %**	**Yield**	**FOS %**	**Yield**	**FOS %**	**Yield**
1	1	-1	-1	-1	1	-1	-1	1	1	-1	1	-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	1	1	-1	-1	-1	1	-1	-1	1	1	-1	1	6.58	16.45	7.71	18.03	8.49	18.35	3.60	7.32
3	1	1	1	-1	-1	-1	1	-1	-1	1	1	-1	11.81	29.52	15.03	32.32	8.63	16.27	8.42	16.51
4	1	1	1	1	-1	-1	-1	1	-1	-1	1	1	0.00	0.00	**20.52**	**49.97**	10.22	21.78	11.2	23.94
5	-1	1	1	1	1	-1	-1	-1	1	-1	-1	1	6.91	34.57	2.98	14.91	0.53	2.63	0.00	0.00
6	1	-1	1	1	1	1	-1	-1	-1	1	-1	-1	0.00	0.00	7.05	14.45	3.55	8.86	3.63	6.65
7	-1	1	-1	1	1	1	1	-1	-1	-1	1	-1	4.86	24.31	0.00	0.00	0.00	0.00	0.00	0.00
8	1	-1	1	-1	1	1	1	1	-1	-1	-1	1	7.68	19.20	12.67	31.78	9.77	24.44	5.46	13.64
9	1	1	-1	1	-1	1	1	1	1	-1	-1	-1	4.00	10.00	9.57	22.60	**22.30**	**54.70**	4.22	9.05
10	-1	1	1	-1	1	-1	1	1	1	1	-1	-1	6.70	33.49	1.63	8.15	1.21	6.09	1.27	6.36
11	-1	-1	1	1	-1	1	-1	1	1	1	1	-1	1.35	6.76	3.87	19.33	2.82	14.09	0.72	3.61
12	1	-1	-1	1	1	-1	1	-1	1	1	1	1	1.42	3.55	**20.34**	**49.34**	16.96	38.16	14.5	30.46
13	-1	1	-1	-1	1	1	-1	1	-1	1	1	1	0,00	0,00	1,00	5,00	0,00	0,00	0,00	0,00
14	-1	-1	1	-1	-1	1	1	-1	1	-1	1	1	0,03	0,14	9,60	45,94	5,94	29,49	3,49	13,76
15	-1	-1	-1	1	-1	-1	1	1	-1	1	-1	1	0,15	0,74	7,05	35,26	3,13	15,64	3,70	18,51
16	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1,05	5,24	6,14	30,73	5,88	29,40	6,51	32,53
PC1	0	0	0	0	0	0	0	0	0	0	0	0	3,62	12,07	8,19	27,29	3,76	12,52	1,61	5,37
PC2	0	0	0	0	0	0	0	0	0	0	0	0	3,62	12,07	8,40	27,96	3,48	11,62	3,69	12,30
PC3	0	0	0	0	0	0	0	0	0	0	0	0	3,62	12,07	9,23	30,76	3,60	12,01	3,34	11,15

A blank experiment without the inouculum, carried out the same conditions as the centre point, demonstrate that the amount of sucrose remained practically stable and none trace of fructtoligosaccharide was formed.

The results showed that high yields reach values of 54.7% at 48h of reaction in T9, 49.97% at 24h in T4 and 49.34% in 24h in T12. The variable effects were estimated for each parameter FOS Yield and total FOS concentration.

For the FOS Yield data showed significance at 24h the variables agitation, with negative effect -16.32 and p-value 0.016; $MnSO_4$, with positive effect 15.33 and p-value 0.020. At 48h the variable was significant only agitation with negative effect -14.94. The inoculum level showed significance at 0h with positive effect 14.08, however it was negative at 24 and 48. Regarding Total FOS Concentration, sucrose levels displayed p-values of 0.0085 at 24h, 0.028 at 48h and 0.046 at 72h. $MnSO_4$ was the only mineral that presenting significant stimulatory effect upon FOS concentration added and urea was included considering alpha 0.2.

The statistical evaluation of the results is shown in Table 3.

Table 3. Estimates of the effects of the parameters analysed at 0, 24 and 48h of the fermentation process

		YIELD (%)				[ ] Total FOS			
Factor	**Time (h)**	**Effect**	**SE**	**t (6)**	**P value**	**Effect**	**SE**	**t (6)**	**P value**
	0	11.59	2.17	5.35	0.00	3.34	0.74	4.54	0.00
Mean	**24**	24.41	2.25	10.85	0.00	7.95	0.90	8.80	0.00
	48	16.63	3.10	5.36	0.00	5.80	1.21	4.82	0.00
	0	-3.32	2.36	-0.70	0.51	1.31	1.60	0.81	0.45
X1	**24**	7.40	4.90	1.51	0.18	**7.58**	**1.97**	**3.85**	**0.01**
	48	10.65	6.76	1.58	0.17	**7.55**	**2.63**	**2.87**	**0.03**
	0	**14.09**	**2.36**	**2.98**	**0.02**	**3.65**	**1.60**	**2.28**	**0.06**
X2	**24**	-9.48	4.90	-1.93	0.10	-1.04	1.97	-0.53	0.62
	48	-5.03	6.76	-0.74	0.48	0.42	2.63	0.16	0.88
	0	7.92	2.36	1.68	0.14	2.05	1.60	1.28	0.25
X3	**24**	6.99	4.90	1.43	0.20	2.69	1.97	1.37	0.22
	48	-4.08	6.76	-0.60	0.57	-1.76	2.63	-0.67	0.53
	0	-3.01	2.36	-0.64	0.55	-1.90	1.60	-1.18	0.28
X4	**24**	4.24	4.90	0.86	0.42	2.20	1.97	1.12	0.31
	48	3.98	6.76	0.59	0.58	2.45	2.63	0.93	0.39
	0	5.78	2.36	1.22	0.27	0.33	1.60	0.20	0.85
X5	**24**	**-16.32**	**4.90**	**-3.33**	**0.02**	**-4.23**	**1.97**	**-2.15**	**0.08**
	48	-14.94	6.76	-2.21	0.07	-4.42	2.63	-1.68	0.14
	0	-3.78	2.36	-0.80	0.45	-0.44	1.60	-0.28	0.79
X6	**24**	-7.94	4.90	-1.62	0.16	-2.78	1.97	-1.41	0.21
	48	2.50	6.76	0.37	0.72	0.79	2.63	0.30	0.77
	0	7.24	2.36	1.53	0.18	2.60	1.60	1.62	0.16
X7	**24**	9.12	4.90	1.86	0.11	3.33	1.97	1.69	0.14
	48	11.21	6.76	1.66	0.15	4.56	2.63	1.73	0.13
	0	-5.45	2.36	-1.15	0.29	-1.60	1.60	-1.00	0.36
X8	**24**	-4.20	4.90	-0.86	0.42	-1.57	1.97	-0.80	0.46
	48	-0.80	6.76	-0.12	0.91	-0.07	2.63	-0.03	0.98
	0	3.24	2.36	0.69	0.52	0.18	1.60	0.11	0.91
X9	**24**	-2.65	4.90	-0.54	0.61	-1.72	1.97	-0.87	0.42
	48	5.89	6.76	0.87	0.42	2.13	2.63	0.81	0.45
	0	-0.37	2.36	-0.08	0.94	0.44	1.60	0.27	0.80
X10	**24**	-1.76	4.90	-0.36	0.73	0.28	1.97	0.14	0.89
	48	-5.62	6.76	-0.83	0.44	-1.23	2.63	-0.47	0.66
	0	-6.92	2.36	-1.47	0.19	-1.70	1.60	-1.06	0.33
X11	**24**	3.25	4.90	0.66	0.53	1.95	1.97	0.99	0.36
	48	-5.04	6.76	-0.75	0.48	-1.29	2.63	-0.49	0.64
	0	-4.33	2.36	-0.92	0.39	-0.88	1.60	-0.55	0.60
X12	**24**	**15.33**	**4.90**	**3.13**	**0.02**	**4.82**	**1.97**	**2.45**	**0.05**
	48	2.64	6.76	0.39	0.71	1.33	2.63	0.51	0.63

For further optimization studies will be considered the factors sucrose, inoculum, urea, $MnSO_4$ concentrations and agitation.

Inoculum Effect: the concretion of inoculums present positive effect only at 0h, that in this time, the action of the enzyme is immediately and major concentration of product is formed. In other times, this showed negative effect, because the synthesis of FOS seems to be related with the growth of *Aureobasidium* and production of the enzyme. However is necessary to study this carefully. The figure 1 presents the values of yield and growth for comparison.

Sucrose Effect: the sucrose certainly is related positively with synthesis of FOS once the activity of the fructosiltransferase and transfructosilation performe with more efficiency in medium with high concentration of the substrate.

Agitation Effect: in this case, showed a negative effect, probably this is explained by the damage caused to the cells when it is accelerate.

Metal ions: In general, metal ions can impact on yeast growth and metabolic processes during fermentation by influencing several important parameters including yeast growth, viability, enzyme activities, stress tolerance, etc. Therefore it is necessary to screen vital mineral nutrients for production of industrially important enzymes. Considering this, the others minerals will not be exclude once they can be essential for the process and will be fix de concentration in the central point.

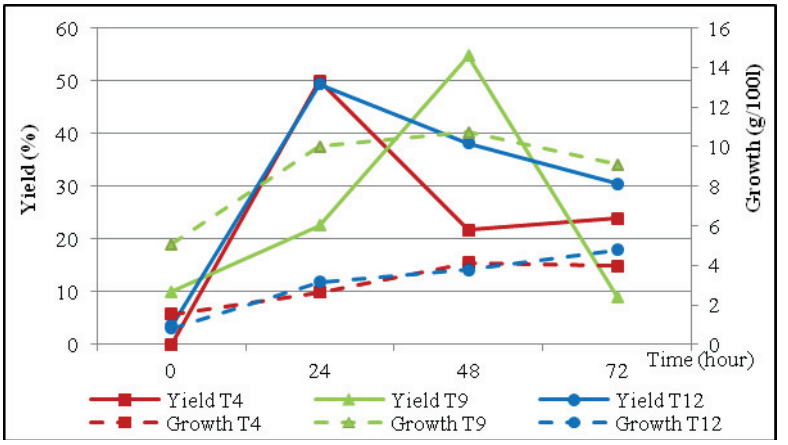

Fig. 1. Experimental values obtained in samples at 0,24, 48 and 72 hoursand simulated time temperature profile

On this study, the highest conversion yield obtained was 54.7% and 223 g/L of FOS total with 400 g/L of initial sucrose concentration. This express the productivity of 4.6 g/l.h and considering T4, the productivity reach value of 8.55 g/l.h.

These results are showing that this process is very promising for the FOS industry considering that the conditions still not have optimized and comparing it with commercial scale of 200 a 300 g/L neoFOS production and with another studies how synthesis of Neo-FOS by *Xantophillomyce dendrorhous* cells that reached 222.72 g/L from 400 g/L of initial sucrose [5]. (Ning et.al., 2010).

The process showed similar results also with biotransformation of sucrose in FOS by *Penicilium expansum* that was directly inoculated in sucrose medium (110 g/L) and presented yield ($Y_{P/S}$) of 0,58 g FOS/g initial sucrose and productitivity 3.25 g/l.h (Prata et.al. 2010).

Therefore, this process was considered to be an added advantage for commercial applications and could be scaled up for commercial production.

4. Conclusion

More research has been done to evaluate and optimize this process. This in turn can provide a focus for effective use of *Aureobasidium pullulans* to synthesize fructooligosaccharide through bioconversion using entire cells to obtain the product by direct manner.

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